

REMARKS

A substitute Sequence Listing is submitted herewith. The substitute Sequence Listing shows sequences that were present in the subject application as filed and, therefore, does not add new matter.

Claims 32, 33 and 78 have been amended. Claims 1-31, 34-35, 38-40, 58-73, 76 were previously canceled without prejudice or disclaimer. Subsequent to the entry of the present amendment, claims 32-33, 36-37, 41-57, 74-75 and 77-78 are pending and at issue. These amendments and additions add no new matter as the amendments are fully supported by the specification and original claims.

I. Amendment to the Claims

Claims 32-33 and 87 have been amended.

Claim 32 has been amended to recite:

A method of identifying an agent that affects isopeptidase activity of a polypeptide comprising:

incubating a test agent with a Rpn11 polypeptide in the presence of a modifier protein and a target protein, wherein the Rpn11 polypeptide has a Jab1/Mpn/Mov34 Metalloenzyme (JAMM) domain consisting essentially of an amino acid sequence of HXHXXXXXXXXXXD (SEQ ID NO:1), wherein H is histidine, D is aspartate, and X is any amino acid, and wherein the polypeptide having isopeptidase activity deconjugates a modifier protein from a target protein by cleaving a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion; and

determining the isopeptidase activity of the test agent by measuring deconjugation of the modifier protein from the target protein in the presence and absence of the test agent, wherein a difference in the isopeptidase activity in the presence versus the absence of the test agent is indicative of an agent that affects isopeptidase activity of the Rpn11 polypeptide, and wherein the Rpn11 polypeptide is as set forth in SEQ ID NO:23 or 24.

Claim 33 has been amended to improve its form and also pursuant to the suggestion on page 6 of the Office Action.

Claim 78 has been amended to recite:

A method of identifying an agent that affects isopeptidase activity of a polypeptide comprising:

incubating a test agent with a AMSH polypeptide in the presence of a modifier protein and a target protein, wherein the AMSH polypeptide has a Jab1/Mpn/Mov34 Metalloenzyme (JAMM) domain consisting essentially of an amino acid sequence of HXHXXXXXXXXXXD (SEQ ID NO:1), wherein H is histidine, D is aspartate, and X is any amino acid, and wherein the AMSH polypeptide having isopeptidase activity deconjugates a modifier protein from a target protein by cleaving a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion; and

determining the isopeptidase activity of the test agent by measuring deconjugation of the modifier protein from the target protein in the presence and absence of the test agent, wherein a difference in the isopeptidase activity in the presence versus the absence of the test agent is indicative of an agent that affects isopeptidase activity of the polypeptide, and wherein the AMSH polypeptide is AMSH as set forth in SEQ ID NO:7.

Claims 32 and 78 have been amended to replace the relevant Accession Nos. for sequence modifiers, or SEQ ID NOs. SEQ ID NO:23 and 24 of claim 32 correspond to Accession Nos.: NP_116659; NP_005796, respectively, which are described on pages 10 and 11 of the specification. SEQ ID NO:7 of claim 78 is the third AMSH polypeptide in the amino acid sequence alignment of FIG.2. SEQ ID NO:7 was also in the originally filed Sequence Listing.

The amendments to the claims do not add new subject matter as they are fully supported by the specification and original claims.

II. Rejections under 35 U.S.C. § 112, Second Paragraph

The claims are variously rejected under 35 U.S.C. §112, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants traverse these rejections as they may apply to the pending claims.

A. Rejection of claims 32 and 78 and dependent claims therefrom

According to the Examiner, “[c]laims 32 and 78 recite the phrase "Accession Nos: NP_116659; NP 005796; and 000487" and "Accession No.: JC7985 and AAD05037". The metes and bounds of these phrase in the context of the claims are not clear. Amino acid sequences of proteins having Accession number can change or be deleted. ... Therefore, Examiner suggests providing SEQ ID NO:, provided applicants have ample support (page 3 of the Office Action)”.

Claims 32 and 78 have been amended to replace the relevant Accession Nos. for sequence modifiers, or SEQ ID NOs. SEQ ID NO:23 and 24 of claim 32 correspond to Accession Nos.: NP_116659; NP_005796, respectively, which are described on pages 10 and 11 of the specification. SEQ ID NO:7 of claim 78 is the third AMSH polypeptide in the amino acid sequence alignment of FIG.2.

A supplemental Sequence Listing adding SEQ ID Nos:23-24 is attached to the end of this paper. This Sequence Listing will replace all previously filed Sequence Listings.

Also, according to the Office Action, “[c]laims 32 and 78 recite the phrase "identifying an agent" and "test agent". It is not clear to the Examiner if the "agent" and "test agent" are the same. If they are the same, Examiner requests amendment of the claims to recite either "agent" or "test agent" to maintain uniformity amongst the claims (page 4 of the Office Action)”.

Claims 32 and 78 have been amended to improve their form. However, the preambles of the claims have not been amended to recite “a test agent” because it is submitted that, in general, “[a] claim is not per se indefinite if the body of the claim recites additional elements which do not appear in the preamble. The mere fact that the body of a claim recites additional elements which do not appear in the claim's preamble does not render the claim indefinite under 35 U.S.C.

112, second paragraph. See *In re Larsen*, No. 01-1092 (Fed. Cir. May 9, 2001) (unpublished)". Hence, the elements in the preamble need not provide the antecedent basis for an element in the body of the claims. So, the first instance of the phrase, "*a* test agent" in the first paragraph of the claims need not have antecedent basis in the preamble because it is the first recitation of the phrase, which then provides antecedent basis for the phrase, "*the* test agent" in the second paragraph of the claims.

Also, according to the Office Action, "[c]laims 32 and 78 recite the phrase "determining the isopeptidase activity agent by measuring deconjugation of the modifier protein from the target protein". The metes and bounds of the phrase in the context of the above claims is not clear to the Examiner. It is not clear to the Examiner as to what applicants mean by "determining the isopeptidase activity agent". Furthermore, it is also not clear as to how those skilled in the art can conclude that the agent identified by the above method modulates isopeptidase activity of Rpn11 or AMSH. It is not clear to the Examiner as to how applicants or those skilled in the art would recognize that said agent being tested is indeed specifically affecting isopeptidase activity of Rpn11 or AMSH and not directly causing the cleavage of the modifier protein from the target protein without affecting the isopeptidase. Therefore, the method lacks essential step(s). Furthermore, since applicants do not indicate that the reaction comprises a target protein which indeed comprises a modifier protein, said target protein and modifier protein lacks antecedence within the a claim. It is also not clear whether the target protein inherently comprises the modifier protein or whether it is added separately (pages 4-5 of the Office Action).

Claims 32 and 78 have been amended to provide antecedent basis for the phrases "modifier protein" and "target protein" in the claims. Claims 32 and 78 have also been amended to replace the phrase, "determining the isopeptidase activity agent" with "determining isopeptidase activity of the test agent". Further, the claims are definite with regards to how one skilled in the art "can conclude that the agent identified by the above method modulates isopeptidase activity of Rpn11 or AMSH (page 4 of the Office Action)". The second paragraph

of claims 32 and 78 recite that the mechanism of cleaving the modifier protein from the target protein by the Rpn11 and/or AMSH is by “measuring deconjugation of the modifier protein from the target protein in the presence and absence of the test agent, wherein a difference in the isopeptidase activity in the presence versus the absence of the test agent is indicative of an agent that affects isopeptidase activity of the polypeptide”; and “deconjugation” is defined and with reference to the first paragraph of claims 32 and 78 which recites that deconjugation is “by cleaving a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion” (see also paragraphs[0029] and [0040]; and Example 1 of the specification). Thus, the claims and the specification unambiguously recite how agents affecting Rpn11 and/or AMSH isopeptidase activity are identified.

Additionally, the Office Action states that, “[c]laims 32 and 78 recite the phrase “determining the isopeptidase activity by measuring deconjugation”. The metes and bounds of the phrase in the context of the above claim is not clear to the Examiner, it is not clear to the Examiner how “measuring deconjugation” of the modifier protein from the target protein leads one of ordinary skill in the art to conclude that a test agent causes a change in isopeptidase activity of Rpn11 or AMSH or what applicants mean by “measuring deconjugation”. This is because the method does not recite steps of distinguishing cleavage of modifier proteins from target proteins in the presence of test agents that reduce isopeptidase activity of Rpn11 or AMSH and test agents that do not modulate isopeptidase activity of Rpn11 or AMSH, since test agents that modulate isopeptidase activity of Rpn11 or AMSH do not ameliorate isopeptidase activity, but can merely reduce isopeptidase activity of Rpn11 or AMSH. Therefore, the method lacks essential step(s) (page 5 of the Office Action)”.

Claims 32 and 78 have been amended to provide antecedent basis for the phrases, “modifier protein” and “target protein” in the claims. The phrase, “measuring deconjugation of the modifier protein from the target protein in the presence and absence of the test agent” in the

second paragraph of the claims refers to the isopeptidase activity of Rpn11 and/or AMSH as recited in the first paragraph of the claims, as discussed above. Thus, one skilled in the art would understand that “measuring deconjugation” is with reference to measuring the “cleav[ed] peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion” as recited in the claims and also as discussed above.

Accordingly, for all the foregoing reasons, withdrawal of rejection of claims 37 and 87 and 38-40, 42-46, 48-66 and 88 under 35 U.S.C. §112, second paragraph is respectfully requested.

B. Rejection of claim 33

According to the Office Action, “[c]laim 33 recites the phrase “an amino acid sequence of SEQ ID NO:2”. It is not clear whether the JAMM domain comprises a fragment of SEQ ID NO:2 or the full length of the amino acid sequence of SEQ ID NO:2 (page 6 of the Office Action).

Claim 33 has been amended to recite “an amino acid sequence of GW(Y/I)H(S/T)HPXXXXXXXXSXXD as set forth in SEQ ID NO. 2”.

Accordingly, withdrawal of rejection of claim 33 under 35 U.S.C. §112, second paragraph is respectfully requested.

C. Rejection of claim 41

According to the Office Action, “[c]laim 41 recites the phrase “not conjugated to the modifier protein”. The metes and bounds of the phrase in the context of the above claim are not clear to the Examiner. It is not clear to the Examiner which protein applicants are referring to. It appears that applicants are actually referring to “target protein deconjugated from the modifier

protein. IF this is so, amending the claim accordingly would overcome this rejection (page 6 of the Office Action)".

The metes and bounds of claim 41 are clear and definite. First, "[t]he modifier protein is conjugated to the target protein via a peptide bond between the carboxy terminus of the modifier protein and a free amino group of the target protein (paragraph [0019] of the specification)". The same is also recited in the first paragraph of independent claims 32 and 78. The claims and the specification are also clear that the claimed assay provides for "deconjugation of a modifier protein from a target protein, e.g., de-neddylation or de-ubiquitination (paragraph 0013)". Thus, the recitation of the phrase, "wherein an increase in the amount of the target protein not conjugated to the modifier protein is indicative of an agent that increases deconjugation of the modifier protein from the target protein" is correct. In short, if one skilled in the art practicing the claimed invention were to see an accumulation of the target protein which is not conjugated (e.g., deubiquitinated) to the modifier protein (e.g., ubiquitin), this is indicative of the test agent being one which increases deconjugation or deubiquitination of the modifier protein from the target protein.

Accordingly, withdrawal of rejection of claim 41 under 35 U.S.C. §112, second paragraph is respectfully requested.

III. Rejections under 35 U.S.C. §112, First Paragraph (written description)

Claims 32-33, 36-37, 41-57, 74-75 and 77 are rejected under on 35 U.S.C. §112, first paragraph for allegedly not containing a written description of the invention and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to practice the method in its best mode. Applicants respectfully traverse the rejection as it applies to the pending claims.

According to the Office Action, “[c]laims 32-33, 36-37,41-57,74-75 and 77 are drawn to a method of identifying an agent that affects isopeptidase activity of a polypeptide, wherein the polypeptide is Rpn11 "Accession Nos.: NP005796 and 000487". However, these polypeptides were not described in the application as originally filed or in any of its parent applications. Therefore, claims 32-33, 36-37,41-57, 74-75 and 77 contain new matter (see page 7 of the Office Action)”.

Independent claim 32 had been amended to recite specific Rpn11 polypeptide “as set forth in SEQ ID NO:23 or 24”, which correspond to Accession Nos.: NP_1166599 and NP_005796, respectively. NP_1166599 is described in the application as filed on page 10, first line under the heading “Eukaryotes”. NP_005796 is described in the application as filed on page 11, second line under the heading “Homo sapiens”.

Also, according to the Office Action, “[c]laims 32-33, 36-37,41-57,74-75 and 77-78encompass a method of using *any or all* Rpn11 complex or AMSH polypeptides and *any* target proteins, including recombinants, mutants and variants, which are isolated from *any* source... The specification only describes a method of identifying agents that modulate isopeptidase activity of a Rpn11 of Accession No. NP116659 by contacting said Rpn11 with ubiquitin and Sic1, as described in Example 1 of the specification... Therefore, the specification fails to describe a representative species of the *genus* comprising *any or all* Rpn11 complex or AMSH, genus comprising *any or all* modifier proteins and genus comprising *any or all* target proteins, including *any or all* recombinants, mutants or variants thereof... (emphasis added; page 8 of the Office Action)”.

First, independent claims 32 and 78 have been amended to recite, for example, SEQ ID NO: 23 and 24, which correspond to Rpn11 polypeptides having Accession Nos.: NP_116659 and NP_005796, respectively; and SEQ ID NO:7, which corresponds to AMSH polypeptide having Accession No. AAD05037. Accession Nos.: NP_116659 and NP_005796 are described

on pages 10 and 11 of the specification; and SEQ ID NO:7 is described as the third AMSH polypeptide in the amino acid sequence alignment of FIG.2. Thus, a “*genus comprising any or all Rpn11 complex or AMSH*” is not recited in the claims, as the claims recite specific Rpn11 and AMSH polypeptides having support in the application as filed.

With regards to a “*genus comprising any or all modifier proteins and genus comprising any or all target proteins, including any or all recombinants, mutants or variants thereof*”, it is submitted that one skilled in the art would understand that “target” and “modifier” proteins are not the limiting factors of the claimed invention. That is, one skilled in the art would understand that the scope of the claimed invention is drawn to a method of identifying an agent affecting Rpn11 or AMSH isopeptidase activity by contacting specific Rpn11 or AMSH polypeptides (SEQ ID NO:23-24), a test agent, in the presence of a target and modifier protein; and further determining the effect of the test agent on the ability of the isopeptidase (Rpn11 or AMSH polypeptides) to cleave the modifier protein from the target protein (Example 1 of the specification).

The present invention also describes the above claimed methods in Example 1. The subject matter of Example 1 was subsequently published in Verma et al. (Oct. 2002) “Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome” *Science* 298(18):611-615 (Exhibit A). Exhibit A discloses the process of protein degradation by 26S proteasomes (page 611, first paragraph):

Proteolysis by the 26S proteasome proceeds by binding of the ubiquitinated substrate protein to the 19S regulatory particle, followed by its unfolding and translocation into the lumen of the 20S core, where it is degraded by the action of the 20S peptidases. At some point in this process, the ubiquitin targeting signal is detached from the substrate.

Example 1 and Exhibit A demonstrate that Rpn11 functions in the absence or presence of a 20S inhibitory agent (e.g., epoxomicin). In the absence of the 20S inhibitory agent, the ubiquitinated substrate (e.g., Sic1) was completely degraded by the 26S proteasome. In contrast, however, in

the presence of the 20S inhibitory agent (e.g., epoxomicin), ubiquitin was removed from the substrate (e.g., Sic1), but the substrate was not degraded any further. Hence, the ubiquitin signal (i.e., modifier protein) was cleaved from the substrate (e.g., Sic1) by the isopeptidase activity in the 26S proteasome (i.e., Rpn11) and not by the peptidase activities associated with the 20S core. Further, mutation of the predicted active-site histidines to alanine (*rpn11AXA*) proved lethal, and co-expression of *rpn11AXA* with wild-type *rpn11* showed that mutant Rpn11AXA or 26S proteasomes were unable to deubiquitinate the substrate (e.g., Sic1), even in the absence of agent (e.g., epoxomicin; paragraph [0075] of the specification; and page FIG.3-4 of Exhibit A). Thus, the claimed invention is sufficiently described in the application as filed.

Moreover, Dr. Craig M. Crews in the attached Declaration states in paragraph 4 that:

The claimed invention is directed to a method of identifying agents which affect Rpn11 or AMSH isopeptidase activity. The method involves detecting the change in Rpn11 or AMSH isopeptidase activity when a test agent is combined with a specific Rpn11 or AMSH polypeptide in the presence of a modifier and target protein. The specific Rpn11 or AMSH polypeptides are described in the application as discussed above, for example, pages 10-11 and FIG.2. Example 1 of the specification describes that the modifier and target proteins are substrates for Rpn11 or AMSH and do not limit the assay, which measures isopeptidase activity of a known or unknown agent. That is, any modifier protein (e.g., ubiquitin) can be used so long as it is a substrate of Rpn11 or AMSH, and Rpn11 or AMSH are capable of cleaving the modifier protein from the associated target protein, in the presence or absence the known or unknown agent (e.g., epoxomicin). Hence, any target protein (e.g., Sic1) can also be used so long as it associates with the modifier protein, and is capable of being cleaved by Rpn11 or AMSH as described in the application. Thus, it is the use of the specific Rpn11 or AMSH polypeptides in the claimed method which is the invention.

Hence, the foregoing demonstrates that Rpn11 or AMSH activity is *independent* of the modifier and/or target protein(s), so long as the modifier protein is a suitable substrate for Rpn11 or AMSH, and the target protein is associated with the modifier protein as discussed above. Thus, the specification sufficiently describes the claimed invention, and one skilled in the art would understand that Applicants were in possession of the same.

Accordingly, withdrawal of rejection of claims 32-33, 36-37, 41-57, 74-75 and 77-78 under 35 U.S.C. §112, first paragraph is respectfully requested.

IV. Rejections under 35 U.S.C. §112, First Paragraph (enablement)

Claims 32-33, 36-37, 41-57, 74-75 and 77-78 are rejected under on 35 U.S.C. §112, first paragraph, for allegedly containing subject matter not described in the specification in such a way as to enable one of skill in the art to make or use the invention. Applicants respectfully traverse the rejection as it applies to the pending claims.

According to the Office Action, “the specification, while being enabling for a method of identifying agents that modulate isopeptidase activity of a Rpn11 of Accession No. NP116659 by contacting said Rpn11 with ubiquitin and Sic1, as described in Example 1 of the specification, does not reasonably provide enablement for a method of identifying agents that modulate isopeptidase activity of Rpn11 complex or AMSH having *any* structure using modifier and target proteins having *any* structure, including *any or all* recombinants, mutants or variants of the same. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims (emphasis added; page 11 of the Office Action)”.

Independent claims 32 and 78 have been amended to recite specific Rpn11 or AMSH polypeptides “as set forth in SEQ ID NO:23 or 24” and “SEQ ID NO:7”. Hence, the claims are not directed to just “any [Rpn11 or AMSH] structure”. The Office Action admits that the specification enables the use of “Rpn11 of Accession No. NP116659 by contacting said Rpn11 with ubiquitin [modifier protein] and Sic1 [target protein]”. As discussed in detail above, the actual “modifier” and “target” proteins do not limit the claimed invention, so long as the “modifier protein is conjugated to the target protein via a peptide bond between the carboxy terminus of the modifier protein and a free amino group of the target protein (paragraph [0019]

of the specification)". The Rpn11 and AMSH polypeptides function by "deconjugate[ing] a modifier protein from a target protein by cleaving a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein in proximity to a metal ion (claims 32 and 78)". Hence, the specification enables one skilled in the art how to make and use the claimed invention.

With regards to the claimed invention being allegedly non- enabling for "using modifier and target proteins having *any* structure, including *any or all* recombinants, mutants or variants of the same (emphasis added; page 11 of the Office Action)", the enclosed Declaration of Dr. Craig M. Crews supports that one skilled in the art would understand that the claimed invention is not about the structure of the modifier and target proteins, rather it is about identifying various test agents which affect the isopeptidase activity of certain Rpn11 and/or AMSH polypeptides (e.g., SEQ ID NO:23-24) which structure and function are described in the application as filed. In order to identify the effect of the test agent on the Rpn11 and/or AMSH, target and modifier proteins (isopeptidase substrates) are used. Please see the Declaration of Dr. Craig M. Crews and the discussion above.

With regards to the specification allegedly being non-enabling for "AMSH having *any* structure", it is submitted that all three of the AMSH polypeptides disclosed in FIG.2 share the conserved JAMM domains as set forth in SEQ ID NO:1 and 2 as claimed in claims 32-33 and 78. The AMSH and Rpn11 polypeptides also share greater homology in the JAMM domain than that set forth in SEQ ID NO:1 and 2, for example, GWYHXHPXXXXXLSXVD (Fig.1 of the specification). McCullough et al. (2004) describe AMSH having deubiquitinating activity *in vitro* by "cleavage of the amide bond between K48 and COOH-terminal glycine of a linked ubiquitin (page 488, col. 1 under "Results and Discussion)". See Exhibit A (McCullough et al. (2004) "AMSH is an endosome-associated ubiquitin isopeptidase" *J Cell Biology* 166(4):487-492) which was attached to the amendment filed April 7, 2005 in response to the Office Action mailed January 12, 2005. In fact, McCullough et al. did not know what the "target protein" for

AMSH was. Hence, McCullough et al. used substrates that were polymers of the "modifier protein", ubiquitin. They used two different polymers that are commercially available: one in which the ubiquitins are linked together via K48 (i.e. lysine residue at amino acid 48 in the sequence of ubiquitin), and the second in which the ubiquitins are linked together via the K63, or the lysine residue at amino acid 63 of ubiquitin. Thus, K48 and K63 define the linkage that joins the modifier protein into a polymer. This method employed by McCullough et al. is a common approach in the field, since such polymers are known to serve as substrates for deconjugating enzymes (e.g., AMSH). Hence, in a case like McCullough et al., there is a proximal and a distal ubiquitin linked via K48 or K63. The proximal ubiquitin refers to the "target" protein that contributes its lysine side chain (either K48 or K63) to the isopeptide bond, whereas distal refers to the "modifier" protein that contributes its carboxy terminus to the isopeptide bond. Thus, McCullough et al. demonstrate that the claimed invention can be reduced to practice as recited, i.e. without naming specific "modifier" and/or "target" proteins, e.g., in McCullough et al. the modifier and target proteins are both ubiquitin.

In short, McCullough et al. reduce to practice the claimed invention, for example, McCullough et al. support that:

[a] method of identifying an agent that affects isopeptidase activity of a polypeptide comprising:

incubating a test agent [e.g., EtOH, TPEN, MeOH etc.,] with a AMSH polypeptide in the presence of a modifier protein [e.g., ub] and a target protein [e.g., ub2, ub4 linked via K48 and K63], wherein the AMSH polypeptide has a Jab1/Mpn/Mov34 Metalloenzyme (JAMM) domain consisting essentially of an amino acid sequence of HXHXXXXXXXXXXD (SEQ ID NO:1), wherein H is histidine, D is aspartate, and X is any amino acid, and wherein the AMSH

polypeptide having isopeptidase activity deconjugates a modifier protein from a target protein by cleaving a peptide bond formed between the carboxy terminus of the modifier protein [e.g., ub] and a free amino group of the target protein [e.g., ub2, ub4 linked via K48 and K63] in proximity to a metal ion [e.g., zinc]; and determining the isopeptidase activity agent by measuring deconjugation of the modifier protein [e.g., ub] from the target protein [e.g., ub2, ub4 linked via K48 and K63] in the presence and absence of the test agent, wherein a difference in the isopeptidase activity in the presence versus the absence of the test agent [e.g., EtOH, TPEN, MeOH etc.,] is indicative of an agent that affects isopeptidase activity of the polypeptide, and wherein the AMSH polypeptide is AMSH as set forth in SEQ ID NO:7. [claim 78]

To summarize the above, the isopeptidase enzyme having a JAMM domain in McCullough et al. is AMSH (Abstract of Exhibit A). The modifier and target proteins in McCullough et al. are ubiquitin and ubiquitin polymers (ub, ub2, ub4; FIG.1 of Exhibit A). The claimed isopeptidase activity of "cleaving a peptide bond formed between the carboxy terminus of the modifier protein and a free amino group of the target protein" is also disclosed by McCullough et al., which states: "the cleavage of the amide bond between K48 and COOH-terminal glycine of a linked ubiquitin (page 488, col. 1 under Results and Discussion)". Therefore, McCullough et al. demonstrate that the claimed invention was reduced to practice essentially as claimed and *first* described by Applicants.

Further, MPEP §2138.05 states that "the inventor need not provide evidence of either conception or actual reduction to practice when relying on the content of the patent application. *Hyatt v. Boone*, 146 F.3d 1348, 1352, 47 USPQ2d 1128, 1130 (Fed. Cir. 1998)....The filing of the original application is, however, evidence of conception of the invention." *In re Costello*, 717 F.2d 1346, 1350, 219 USPQ 389, 392 (Fed. Cir. 1983)". So, although Applicants may not have described an example demonstrating AMSH isopeptidase activity as claimed, Applicants can

In re Application of:

Cope et al.

Application No.: 10/047,253

Filed: January 14, 2002

Page 21

PATENT

Attorney Docket No.: CIT1510-4

“[rely] on the content of the patent application” as “evidence of conception of the invention”. MPEP §§2138.05. Hence, Applicants have described AMSH proteins with JAMM domains, having isopeptidase activity which cleaves the modifier protein from the target protein as claimed and as shown by McCullough et al. Thus, as stated in MPEP §2138.05, the “filing of [Applicants’] patent application serves as conception and constructive reduction to practice of the subject matter described in the application,” and Applicants were the first to describe the claimed invention regarding either Rpn11 or AMSH polypeptides.

Therefore, the foregoing demonstrates that the claimed invention enables one skilled in the art to make and use the claimed invention as described in the application.

Accordingly, withdrawal of rejection of claims 32-33, 36-37, 41-57, 74-75 and 77-78 under 35 U.S.C. §112, first paragraph is respectfully requested.

In re Application of:

Cope et al.

Application No.: 10/047,253

Filed: January 14, 2002

Page 22

PATENT

Attorney Docket No.: CIT1510-4


CONCLUSION

In view of the amendments and above remarks, it is submitted that the claims are in condition for allowance, and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicant's undersigned representative if there are any questions relating to this application.

Check Number 582117 in the amount of \$60.00 is enclosed for the One-Month Extension of Time Fee. No other fee is deemed necessary with the filing of this paper. However if any fees are due, the Commissioner is hereby authorized to charge any fees, or make any credits, to Deposit Account No. 07-1896 referencing the above-identified attorney docket number. A copy of the Transmittal Sheet is enclosed.

Respectfully submitted,

Date: June 8, 2006

for  Reg 38,724
Lisa A. Haile, J.D., Ph.D.
Registration No. 38,347
Telephone: (858) 677-1456
Facsimile: (858) 677-1465

DLA PIPER RUDNICK GRAY CARY US LLP
4365 Executive Drive, Suite 1100
San Diego, California 92121-2133
USPTO Customer No. 28213

of ubiquitin conjugates from proteasome substrates (32). There exist other Csn5/Rpn11 homologs in eukaryotes (Fig. 1) and, by extension, we propose the "JAMMIN" hypothesis, which posits that eukaryotic JAMM proteins are isopeptidases that deconjugate Nedd8 or other ubiquitin-like proteins.

Drosophila sustained by Csn5 carrying a JAMM domain mutation arrest development as larvae with abnormalities in photoreceptor differentiation, suggesting that at least two functions associated with Csn5—viability and photoreceptor differentiation—require its JAMM-dependent isopeptidase activity. Given that Csn5 has been implicated in c-jun signaling (12), p27 turnover (33), cytokine signaling (14), and growth cone-target interactions (11), it will be interesting to see if isopeptidase activity of Csn5 underlies these diverse processes as well.

All neddylated proteins known are members of the cullin family. It is not clear whether CSN isopeptidase acts exclusively upon cullin-Nedd8 conjugates or cleaves other targets. Regardless, given the large number of F-box proteins and the potential for substantial diversity in the substrates for SCF and other cullin-based ubiquitin ligases, CSN deneddylase activity may play an enormous role in cellular regulation.

References and Notes

- D. Skowrya, K. L. Craig, M. Tyers, S. J. Elledge, J. W. Harper, *Cell* **91**, 209 (1997).
- R. M. Feldman, C. C. Correll, K. B. Kaplan, R. J. Deshaies, *Cell* **91**, 221 (1997).
- R. J. Deshaies, *Annu. Rev. Cell Dev. Biol.* **15**, 435 (1999).
- F. Osaka et al., *EMBO J.* **19**, 3475 (2000).
- T. Kawakami et al., *EMBO J.* **20**, 4003 (2001).
- S. Lyapina et al., *Science* **292**, 1382 (2001).
- C. Schwechheimer et al., *Science* **292**, 1379 (2001).
- M. Seeger, C. Gordon, W. Dubiel, *Curr. Biol.* **11**, R643 (2001).
- N. Wei, X. W. Deng, *Trends Genet.* **15**, 98 (1999).
- S. Freilich et al., *Curr. Biol.* **9**, 1187 (1999).
- G. S. Suh et al., *Neuron* **33**, 35 (2002).
- F. X. Claret, M. Hibi, S. Dhut, T. Toda, M. Karin, *Nature* **383**, 453 (1996).
- R. Kleemann et al., *Nature* **408**, 211 (2000).
- E. Bianchi et al., *Nature* **404**, 617 (2000).
- G. Cope, R. Deshaies, unpublished data.
- C. Zhou et al., *BMC Biochem.* [online] **2**, article #7 (2001). Available at: www.biomedcentral.com/bmcbiochem.
- S. F. Altshul et al., *Nucleic Acids Res.* **25**, 3389 (1997).
- L. Aravind, C. P. Ponting, *Protein Sci.* **7**, 1250 (1998).
- K. E. Mundt et al., *Curr. Biol.* **9**, 1427 (1999).
- C. P. Ponting, L. Aravind, J. Schultz, P. Bork, E. V. Koonin, *J. Mol. Biol.* **289**, 729 (1999).
- N. D. Rawlings, A. J. Barrett, *Methods Enzymol.* **248**, 183 (1995).
- Like JAMM, metal-dependent peptidases often possess two conserved acidic residues: one that acts as a ligand for zinc and a second that acts as a nucleophile to promote attack by water on the peptide backbone. Based on the common occurrence of Glu as a nucleophile and on the predicted secondary structure of the Jab1/MPN domain, we propose that the Glu helps polarize a water molecule for hydrolytic attack and the COOH-terminal His and Asp residues serve as three ligands for zinc.
- N. Wei, X. W. Deng, *Photochem. Photobiol.* **68**, 237 (1998).
- A. G. Loewy et al., *J. Biol. Chem.* **268**, 9071 (1993).
- D. R. Holland, A. C. Hausrath, D. Juers, B. W. Matthews, *Protein Sci.* **4**, 1955 (1995).
- M. Gomez-Ortiz, F. X. Gomis-Ruth, R. Huber, F. X. Aviles, *FEBS Lett.* **400**, 336 (1997).
- D. Lammer et al., *Genes Dev.* **12**, 914 (1998).
- D. Liakopoulos, G. Doenges, K. Matuschewski, S. Jentsch, *EMBO J.* **17**, 2208 (1998).
- A. C. Gavin et al., *Nature* **415**, 141 (2002).
- V. N. Podust et al., *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4579 (2000).
- Null mutant survival up to third instar larvae was context dependent. Although Csn5-null larvae rarely survived to third instar at UCLA, we routinely found that these mutants survived to third instar at CalTech.
- R. Verma et al., *Science* **298**, 611 (2002).
- K. Tomoda, Y. Kubota, J. Kato, *Nature* **398**, 160 (1999).
- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- C. Notredame, D. G. Higgins, J. Heringa, *J. Mol. Biol.* **302**, 205 (2000).
- B. Rost, C. Sander, R. Schneider, *Comput. Appl. Biosci.* **10**, 53 (1994).
- Materials and Methods are available as supporting material on Science Online.
- We thank D. Wolf, M. Hochstrasser, K. Mundt, and A. Carr for generously providing yeast strains and plasmids, the S. Benzer lab for providing lab equipment and space, and S. Lyapina for psCSN. We thank members of the Deshaies lab for providing helpful insight and discussions. This work was supported by NIH (G.A.C.) and the Howard Hughes Medical Institute.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1075901/DC1
Materials and Methods
Figs. S1 and S2
References and Notes

9 July 2002; accepted 7 August 2002

Published online 15 August 2002;

10.1126/science.1075901

Include this information when citing this paper.

Role of Rpn11 Metalloprotease in Deubiquitination and Degradation by the 26S Proteasome

Rati Verma,¹ L. Aravind,² Robert Oania,¹ W. Hayes McDonald,³ John R. Yates III,³ Eugene V. Koonin,² Raymond J. Deshaies^{1*}

The 26S proteasome mediates degradation of ubiquitin-conjugated proteins. Although ubiquitin is recycled from proteasome substrates, the molecular basis of deubiquitination at the proteasome and its relation to substrate degradation remain unknown. The Rpn11 subunit of the proteasome lid subcomplex contains a highly conserved Jab1/MPN domain-associated metalloisopeptidase (JAMM) motif—EX_nHXXH₁₀D. Mutation of the predicted active-site histidines to alanine (*rpn11AXA*) was lethal and stabilized ubiquitin pathway substrates in yeast. Rpn11^{AXA} mutant proteasomes assembled normally but failed to either deubiquitinate or degrade ubiquitinated Sic1 in vitro. Our findings reveal an unexpected coupling between substrate deubiquitination and degradation and suggest a unifying rationale for the presence of the lid in eukaryotic proteasomes.

Proteolysis by the 26S proteasome proceeds by binding of the ubiquitinated substrate protein to the 19S regulatory particle, followed by its unfolding and translocation into the lumen of the 20S core, where it is degraded by the action of the 20S peptidases (1–3). At some point in this process, the ubiquitin targeting signal is detached from the substrate. It is appealing to envision that this deubiquitination is obligatorily coupled to degradation. Such coupling would render the targeting event irreversible, prevent unproductive turn-

over of ubiquitin, and presumably alleviate steric blockade of the 20S core entry portal by the bulky ubiquitin chain, which is linked by isopeptide bonds. When and where substrate deubiquitination takes place, the identity of the deubiquitinating enzyme (DUB), and whether deubiquitination of a substrate is essential for its degradation by the proteasome are unclear (4, 5).

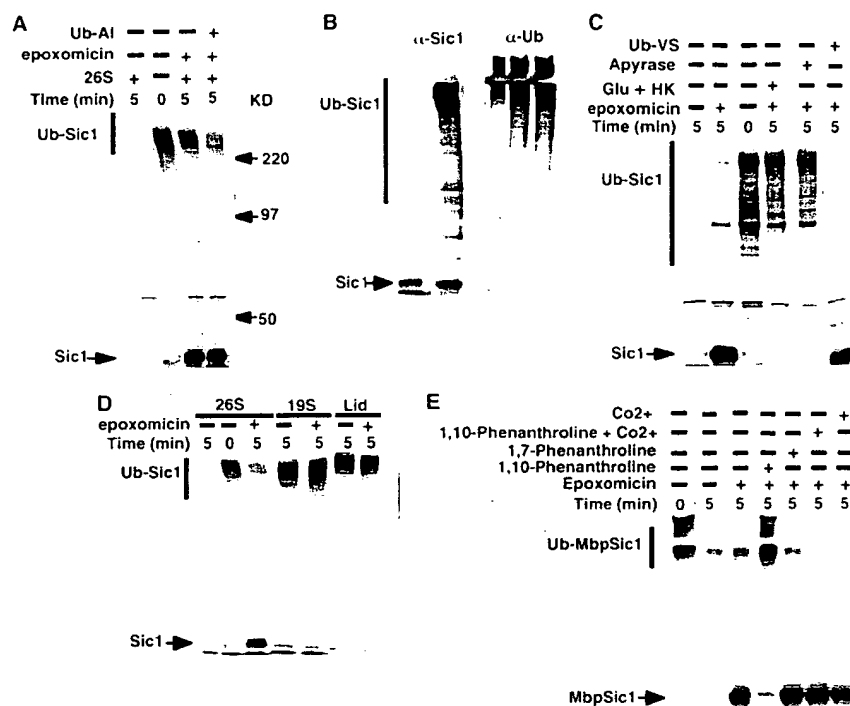
Budding yeast ubiquitinated S-Cdk inhibitor Sic1 (Ub-Sic1) is rapidly degraded by purified 26S proteasomes (3, 6) in a reaction that recapitulates physiological requirements for Sic1 proteolysis (7, 8). To investigate whether degradation of Sic1 is normally accompanied by its deubiquitination, we evaluated the fate of Ub-Sic1 after inhibition of 26S proteolytic activity. Epoxomicin inhibits the proteasome by covalently binding the catalytically active β subunits of the 20S core (9). Purified 26S proteasomes were preincu-

¹Department of Biology and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125, USA. ²National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA. ³Department of Cell Biology, The Scripps Research Institute, San Diego, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: deshaies@caltech.edu

REPORTS

Fig. 1. Characterization of Ub-Sic1 deubiquitination by purified 26S proteasomes. (A) Ub-Sic1 is deubiquitinated by epoxomicin-treated 26S proteasomes in a ubiquitin aldehyde-insensitive manner. Purified proteasomes (100 nM) were incubated with either 1% dimethyl sulfoxide (DMSO) (lanes 1 and 2, mock), or 100 μ M epoxomicin in the absence (lane 3) or presence (lane 4) of 2.5 μ M ubiquitin aldehyde (Ub-Al) for 30 min at 30°C. The degradation reaction was initiated by the addition of Ub-Sic1 (300 nM) and 1 \times ATP regenerating system (1 \times ARS) (3), incubated at 30°C for 5 min, and terminated by the addition of SDS sample buffer. Aliquots were resolved by SDS-PAGE (8% polyacrylamide gel), transferred to nitrocellulose, and immunoblotted with polyclonal antibody to Sic1 to monitor degradation. (B) Sic1 generated by epoxomicin-treated proteasomes is completely deubiquitinated. An aliquot of the epoxomicin-treated sample from lane 3 in (A) as well as a Ub-Sic1 preparation containing both Ub-Sic1 and unmodified Sic1 were resolved by SDS-PAGE and immunoblotted with antiserum to Sic1 (α -Sic1) (left) or to ubiquitin (α -Ub) (right). The anti-ubiquitin immunoreactivity is primarily derived from autoubiquitinated Cdc34 (30), which is not degraded (37). (C) Deubiquitination of Ub-Sic1 is ATP dependent. Mock- or epoxomicin-treated 26S proteasomes were depleted of ATP by 5 min of incubation with apyrase (15 units/ml) or hexokinase (5 units/ml) plus 30 mM glucose (Glu + HK) before incubation with Ub-Sic1. Lane 6 is the same as lane 2, except the DUB inhibitor ubiquitin vinyl sulfone (Ub-VS) (32) was included at 2.5 μ M. (D) Subparticles of the 26S proteasome do not efficiently deubiquitinate Ub-Sic1. The 19S regulatory particle was isolated as described in (6). The lid subparticle of 19S was purified from a strain containing *RPN8TEV2MYC9* as described (23). A Coomassie blue-stained preparation is shown in Fig. 4C. The three preparations—26S, 19S, and lid—were incubated in the absence (lanes 1, 2, 4, and 6) or presence (lanes 3, 5, and 7) of epoxomicin and assayed for degradation/



deubiquitination of Ub-Sic1 as described in (A). (E) Inhibition of deubiquitination by the metal chelator 1,10-phenanthroline. 26S proteasomes were preincubated at 30°C with 1% DMSO and 1% MeOH (lanes 1 and 2) or 100 μ M epoxomicin and 1% MeOH (lane 3) containing in addition 1 mM 1,10-phenanthroline (lane 4), 1 mM 1,7-phenanthroline (lane 5), or 1 mM 1,10-phenanthroline premixed with 2 mM CoCl_2 (lane 6) or 2 mM CoCl_2 (lane 7). The degradation reaction was initiated by the addition of Ub-MbpSic1.

A

COP9_au5_Hs	NLE.....VGRLENAIGWYHSHPGYGCWLSGIDVSTQMLNQFPQEPFVA--VVIDPTRTISAGKVN
Rpn11_Sc	PME.....TGRDQMVVGWYHSHPGFGLSSVDVNTQKSFQELNSRAVA--VVVDPIQSVKG-KVV
Pad1_Sp	PME.....TGRPEMVVGWYHSHPGFGLSSVDINTQSFQELTPRAVA--VVVDPIQSVKG-KVV
Rpn11_C.e1e	PME.....TGRPEMVVGWYHSHPGFGLSSVDINTQSFQELSDRAVA--VVVDPIQSVKG-KVV
Poh1_Hs	PME.....TGRPEMVVGWYHSHPGFGLSSVDINTQSFQELSERAVA--VVVDPIQSVKG-KVV
.....E(X)n.....HXHXXXXXXXXXXD

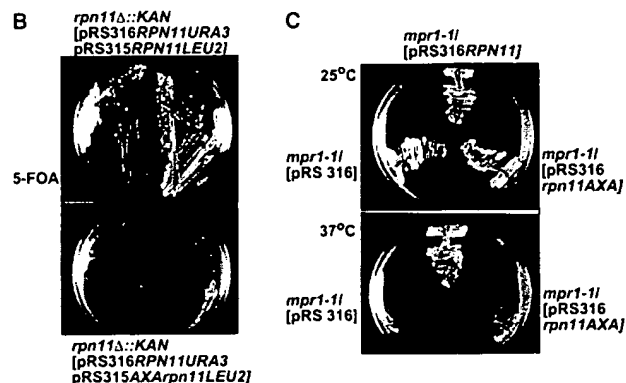


Fig. 2. Rpn11, an intrinsic subunit of the lid subcomplex of the 19S regulatory particle, contains a conserved, predicted metalloprotease motif (JAMM) that is critical for viability. (A) Sequence alignment of *RPN11* orthologs with human CSN5/JAB1 reveals the JAMM motif. The multiple alignment was constructed by using the T-Coffee program (33). Each protein is denoted by its name followed by an abbreviated species name. The predicted metal-chelating and catalytic residues are shown as a consensus below the alignment. For a detailed description of the extended JAMM domain consensus based on all *RPN11* orthologs, see fig. S3. (B) An intact JAMM motif is critical for yeast cell proliferation. An *rpn11Δ::KAN* strain kept alive by an [*RPN11 URA3*] plasmid (RJD 1922) was transformed with *LEU2*-marked *RPN11* (RJD 1934) or *rpn11AXA* plasmids (RJD 1935) (23). Transformants (two representative clones each) were streaked onto 5-fluoroorotic acid (FOA) plates and allowed to grow for 5 days at 25°C. Because 5-FOA is toxic to *URA3* cells, growth is observed only when the *URA3*-marked plasmid is rendered dispensable by the *LEU2* plasmid. (C) The *rpn11AXA* mutant cannot complement the *mpr1-1* temperature-sensitive allele of *RPN11*. *mpr1-1* (RJD 1786) was transformed with either empty pRS316 [*URA3*] vector or vector containing wild-type *RPN11* (RJD 1815) or *rpn11AXA* (RJD 1816). Individual transformants were streaked on synthetic minimal (SD)-Ura plates and incubated at 25°C or 37°C for 5 days.

bated with or without epoxomicin, Ub-Sic1 was then added, and degradation was monitored by the loss of Sic1 antigen. In the absence of epoxomicin, Ub-Sic1 was completely degraded (Fig. 1A; fig. S1). Surprisingly, in the presence of epoxomicin, a

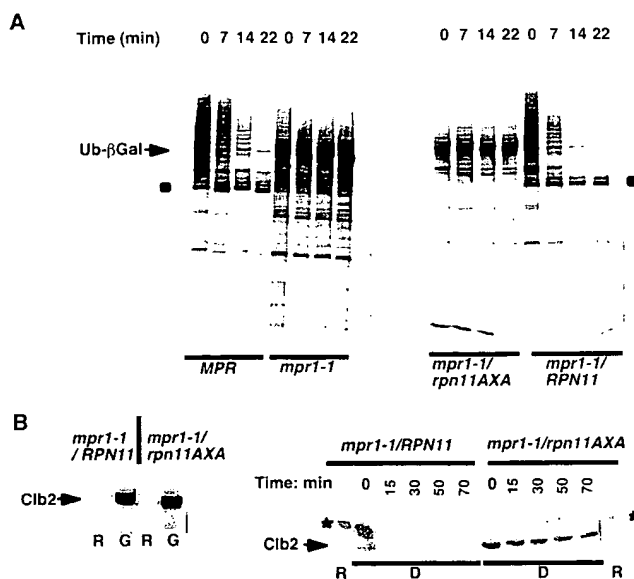
large fraction of Ub-Sic1 molecules were completely deubiquitinated, as judged by comigration with unmodified Sic1 and failure to cross-react with antibodies to ubiquitin (Fig. 1B).

Conversion of Ub-Sic1 to Sic1 differed

from conventional thiol protease-mediated deubiquitination in four respects: (i) it was insensitive to the classic DUB inhibitors ubiquitin aldehyde (Ub-Al) and ubiquitin vinyl sulfone (Ub-VS) (Fig. 1, A and C); (ii) it required adenosine triphosphate (ATP) (Fig.

REPORTS

Fig. 3. An intact JAMM motif is required for degradation of ubiquitin-proteasome pathway substrates in vivo. (A) Stabilization of a UFD pathway substrate in mutant cells. *RPN11* (RJD1901), *mpr1-1* (RJD1902), *mpr1-1 RPN11* (RJD1903), and *mpr1-1 rpn11AXA* (RJD1904) strains (23) containing a reporter plasmid expressing the unstable Ub-V76-Val-eK-B-Gal fusion protein were analyzed by pulse-chase ^{35}S radiolabeling as described in (6). Arrow indicates the ^{35}S -labeled substrate protein, and filled circle shows the position of the 90-kD stable breakdown product of the reporter protein. (B) Stabilization of mitotic cyclin Clb2 in *mpr1-1 rpn11AXA* cells. Mutant *mpr1-1 RPN11* (RJD2002) and *mpr1-1/rpn11AXA* (RJD2004) strains harboring a chromosomally encoded, influenza hemagglutinin antigen (HA)-tagged *GAL-CLB2* expression cassette (23) were grown in raffinose medium (R) at 25°C, and Clb2 expression was induced by the addition of 2% galactose (G). To confirm specific expression of Clb2-HA3, we prepared cell lysates by lysing with glass beads and boiling. Aliquots were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with 12CA5 antibody to HA ascites (left). For evaluation of Clb2-HA3 stability (right), cultures were grown in raffinose (R) medium at 25°C and arrested in G_1 by the addition of α -factor (5 $\mu\text{g}/\text{ml}$) for 2 hours. Clb2 expression was induced with galactose for 1.5 hours, and cultures were shifted to 36°C to inactivate *mpr1-1*. After 1.5 hours, cells were transferred to α -factor-containing dextrose (D) medium at 37°C to extinguish expression of Clb2. Because there is continuous proteolysis of Clb2 in α -factor-arrested cells, and time 0 was collected after washing and resuspension in yeast extract, peptone, and dextrose, the level is much less than that seen in exponential by growing cells (left). Asterisk denotes a nonspecific immunoreactive band to HA.



REPORTS

plexes in vitro. By contrast, proteasomes recovered from both *mpr1-1/RPN11* and *mpr1-1/rpn11AXA* cells were assembled into functional particles as judged by native gel electrophoresis (Fig. 4E), denaturing SDS-PAGE (Fig. 4B), and immunoblot analysis (Fig. 4D) and contained all 26S subunits as determined by mass spectrometry (table S1).

The "isogenic" Rpn11- and Rpn11^{AXA}-containing 26S proteasomes were next tested for their ability to deubiquitinate and degrade Ub-Sic1. As expected, Rpn11-containing 26S proteasomes degraded Ub-Sic1, and preincubation with epoxomicin blocked degradation,

leading to the accumulation of deubiquitinated Sic1 (Fig. 4F). In contrast, the Rpn11^{AXA} 26S proteasomes were profoundly defective for both activities. This result suggests a compelling biochemical rationale for the lethality of *rpn11AXA*.

We envision the following sequence of steps in the degradation cycle. After binding of a multiubiquitinated substrate to the 26S proteasome, the substrate is unfolded and threaded into the 20S core. Concomitantly, the substrate is deubiquitinated by the metalloisopeptidase activity of Rpn11. If Rpn11 activity is blocked, we envision that the tetraubiquitin chain targeting signal, which has a diameter of about 28 Å

(as deduced from the crystal structure coordinates in the Protein Data Bank), would sterically block further substrate translocation upon collision with the entry portal of the 20S proteasome, which has a diameter comparable to that reported for *Thermoplasma acidophilum* (13 Å) (25). Substrate deubiquitination by Rpn11 thus defines a new, key step in protein degradation by 26S proteasome. Because deubiquitination of Ub-Sic1 requires ATP and is not sustained by a free lid or 19S regulatory particle, we propose that deubiquitination of a substrate is normally coupled tightly to its translocation into the 20S core. Although the mechanism underlying this coupling is unknown, we posit that it renders degradation vectorial by preventing deubiquitination until the substrate is irreversibly committed to proteolysis.

Eukaryotic proteasomes are distinguished from bacterial and archaeal ATP-dependent proteases primarily by their dependence on ubiquitin and the presence of the lid subcomplex (26). Highly specific targeting of substrates can be mediated by prokaryotic adenosine triphosphatases (ATPases) (27, 28), and Rpt5 ATPase appears to be the proteasomal receptor for ubiquitin chains (29). Taken together, these observations raise the question of why eukaryotic proteasomes have a lid. A unifying simplification emerges if one considers that a major—and perhaps only—function of the lid is to serve as a specialized isopeptidase that tightly couples the deubiquitination and degradation of substrates.

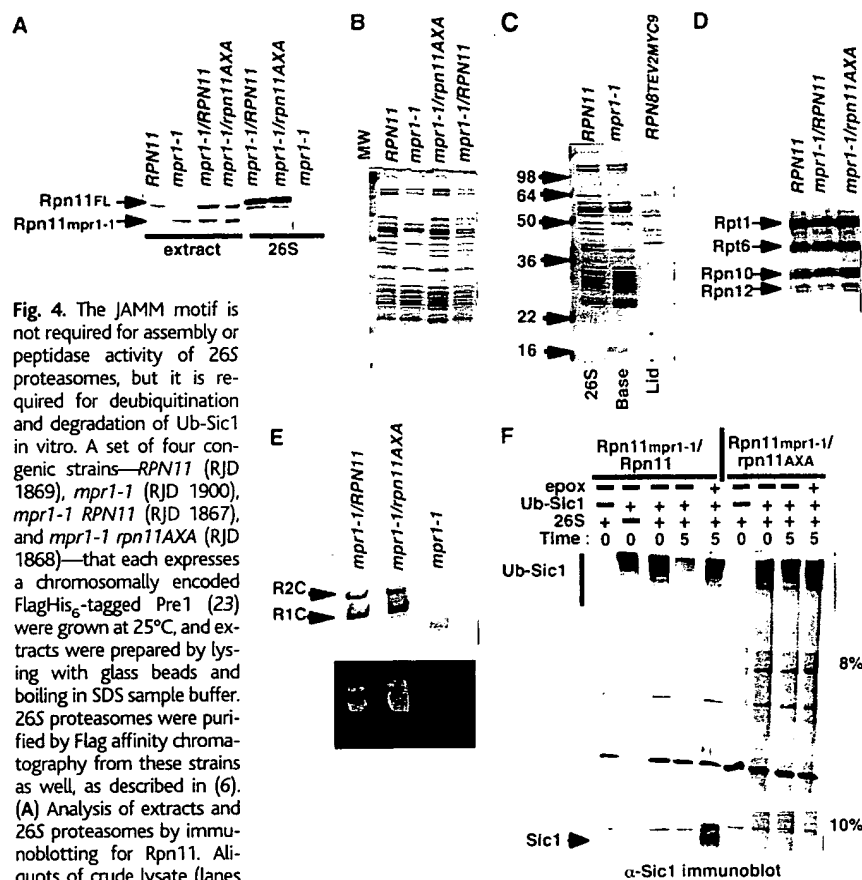


Fig. 4. The JAMM motif is not required for assembly or peptidase activity of 26S proteasomes, but it is required for deubiquitination and degradation of Ub-Sic1 in vitro. A set of four congeneric strains—*RPN11* (RJD 1869), *mpr1-1* (RJD 1900), *mpr1-1 RPN11* (RJD 1867), and *mpr1-1 rpn11AXA* (RJD 1868)—that each expresses a chromosomally encoded FlagHis₆-tagged Pre1 (23) were grown at 25°C, and extracts were prepared by lysing with glass beads and boiling in SDS sample buffer. 26S proteasomes were purified by Flag affinity chromatography from these strains as well, as described in (6). (A) Analysis of extracts and 26S proteasomes by immunoblotting for Rpn11. Aliquots of crude lysate (lanes 1 to 4) and purified proteasomes (lanes 5 to 7) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiserum to Pad1, the *Schizosaccharomyces pombe* Rpn11 ortholog (24). (B and C) *mpr1-1* but not *rpn11AXA* mutation alters polypeptide composition of 26S proteasome. Proteasomes isolated from the indicated strains were fractionated by SDS-PAGE and stained with Coomassie blue. The lid subparticle shown in (C) was purified as described in (23). (D) *rpn11AXA* mutation does not perturb assembly of the 19S regulatory particle. Purified 26S proteasomes were evaluated by immunoblotting with antibodies specific for the indicated proteasome subunits. (E) The *mpr1-1* but not *rpn11AXA* mutation compromises assembly and peptidase activity of 26S proteasome. Purified 26S proteasomes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel and analyzed by Coomassie blue staining (top) or in-gel peptidase activity by incubating with the fluorescent peptidase substrate Suc-LLVY-AMC, as described in (6). (F) Point-mutant proteasomes with an altered JAMM motif are unable to deubiquitinate and degrade Ub-Sic1 in vitro. 26S proteasomes were prepared by anti-Flag affinity chromatography from RJD 1867 (*mpr1-1 RPN11*) and RJD 1868 (*mpr1-1 rpn11AXA*) cells expressing a chromosomally encoded FlagHis₆-tagged Pre1. Purified proteasomes were incubated with 2.5 μM ubiquitin aldehyde at 30°C in the presence or absence of 100 μM epoxomicin. Ub-Sic1 and ATP-regenerating system were then added, and degradation was monitored as described in Fig. 1. Lanes 1 and 6 contain the 26S proteasome preparations from *mpr1-1 RPN11* and *mpr1-1 rpn11AXA*, respectively, with no Ub-Sic1 added. The same samples were run on an 8% gel (top) as well as a 10% gel (bottom) to better visualize the Ub-Sic1 conjugates and unmodified Sic1.

References and Notes

1. A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **67**, 425 (1998).
2. J. Thrower, L. Hoffman, M. Rechsteiner, C. Pickart, *EMBO J.* **19**, 94 (2000).
3. R. Verma, H. McDonald, J. R. Yates III, R. J. Deshaies, *Mol. Cell* **8**, 439 (2001).
4. K. D. Wilkinson, M. Hochstrasser, in *Ubiquitin and the Biology of the Cell*, J. Peters, J. Harris, D. Finley, Eds. (Plenum Press, New York, 1998), pp. 99–125.
5. C. H. Chung, S. H. Baek, *Biochem. Biophys. Res. Commun.* **266**, 633 (1999).
6. R. Verma et al., *Mol. Biol. Cell* **11**, 3425 (2000).
7. E. Schwob, T. Böhm, M. Mendenhall, K. Nasmyth, *Cell* **79**, 233 (1994).
8. R. Verma et al., *Science* **278**, 455 (1997).
9. L. Meng et al., *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10403 (1999).
10. E. Eytan, T. Armon, H. Heller, S. Beck, A. Hershko, *J. Biol. Chem.* **268**, 4668 (1993).
11. M. Glickman et al., *Cell* **94**, 615 (1998).
12. S. Lyapina et al., *Science* **292**, 1382 (2001).
13. C. P. Ponting, L. Aravind, J. Schultz, P. Bork, E. V. Koonin, *J. Mol. Biol.* **289**, 729 (1999).
14. G. A. Cope et al., *Science* **298**, 608 (2002).
15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
16. T. Rinaldi, C. Ricci, D. Porro, M. Bolotin-Fukuhara, L. Frontali, *Mol. Biol. Cell* **9**, 2917 (1998).
17. R. C. Conaway, C. S. Brower, J. W. Conaway, *Science* **296**, 1254 (2002).
18. M. L. Stitzel, R. Durso, J. C. Reese, *Genes Dev.* **15**, 128 (2001).
19. E. S. Johnson, B. Bartel, W. Seufert, A. Varshavsky, *EMBO J.* **11**, 497 (1992).

20. A. Amon, S. Irniger, K. Nasmyth, *Cell* **77**, 1037 (1994).
21. W. Seufert, S. Jentsch, *EMBO J.* **11**, 3077 (1992).
22. F. R. Papa, M. Hochstrasser, *Nature* **366**, 313 (1993).
23. Materials and methods are available as supporting material on Science Online.
24. M. Shimanuki, Y. Saka, M. Yanagida, T. Toda, *J. Cell Sci.* **108**, 569 (1995).
25. M. Groll et al., *Nature Struct. Biol.* **7**, 1062 (2000).
26. D. Voges, P. Zwickl, W. Baumeister, *Annu. Rev. Biochem.* **68**, 1015 (1999).
27. C. K. Smith, T. A. Baker, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6678 (1999).
28. R. Verma, R. J. Deshaies, *Cell* **101**, 341 (2000).
29. Y. A. Lam, T. G. Lawson, M. Velayutham, J. L. Zweier, C. M. Pickart, *Nature* **416**, 763 (2002).
30. J. Seol et al., *Genes Dev.* **13**, 1614 (1999).
31. R. Verma, unpublished observations.
32. A. Borodovsky et al., *EMBO J.* **20**, 5187 (2001).
33. C. Notredame, D. G. Higgins, J. Heringa, *J. Mol. Biol.* **302**, 205 (2000).
34. This work was supported by Howard Hughes Medical Institute (R.V. and R.J.D.) and by a grant to University of Washington (W.H.M. and J.R.Y., UOW/RR11823-05-01). We are indebted to C. Crews for epoxomicin, K. Takeda and T. Toda for antiserum to pad1/Rpn11, T. Rinaldi for the MPR and mpr1-1 strains, M. Hochstrasser for ubp6Δ, C. H. Chung for Ubp6 antiserum,

G. Alexandru and K. Nasmyth for the GAL-CLB2-HA3 strain, and the Deshaies lab members for critical comments.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1075898/DC1

Materials and Methods

Figs. S1 to S3

Tables S1 to S3

9 July 2002; accepted 7 August 2002

Published online 15 August 2002;

10.1126/science.1075898

Include this information when citing this paper.

Impacts of Soil Faunal Community Composition on Model Grassland Ecosystems

M. A. Bradford,^{1*} T. H. Jones,² R. D. Bardgett,³ H. I. J. Black,⁴ B. Boag,⁵ M. Bonkowski,⁶ R. Cook,⁷ T. Eggers,¹ A. C. Gange,⁸ S. J. Grayston,⁹ E. Kandeler,¹⁰ A. E. McCaig,^{11†} J. E. Newington,¹ J. I. Prosser,¹¹ H. Setälä,¹² P. L. Staddon,^{13‡} G. M. Tordoff,¹ D. Tschirko,¹⁰ J. H. Lawton^{1,14}

Human impacts, including global change, may alter the composition of soil faunal communities, but consequences for ecosystem functioning are poorly understood. We constructed model grassland systems in the Ecotron controlled environment facility and manipulated soil community composition through assemblages of different animal body sizes. Plant community composition, microbial and root biomass, decomposition rate, and mycorrhizal colonization were all markedly affected. However, two key ecosystem processes, aboveground net primary productivity and net ecosystem productivity, were surprisingly resistant to these changes. We hypothesize that positive and negative faunal-mediated effects in soil communities cancel each other out, causing no net ecosystem effects.

Soil fauna are essential to efficient nutrient cycling, organic matter turnover, and maintenance of soil physical structure, processes that are key determinants of primary production and ecosystem carbon storage (1–3). Consequently, there is considerable concern about impacts on ecosystem functioning that might result from shifts in the community composition of soil fauna mediated through global change (4–6). Predictions based on theoretical considerations of soil communities are ambivalent. Indeterminate and unexpected impacts are predicted from food web theory (7, 8). Redundancy is also postulated to be common (9), with large changes in community composition having minimal effects. Anderson (10) argued that net effects may be positive, negative, or zero, depending on the balance between sink and source processes operating at finer scales. Keystone species theory (11) and distinct bacterial versus fungal energy channels (12, 13) further cloud the predictions. Therefore, an empirical approach is essential for predicting the impacts of shifts in soil community composition on ecosystem functioning.

Pot experiments with soil, soil organisms, and sometimes an individual plant or plant species have demonstrated the marked potential effects of loss of specific soil fauna and faunal groups on a range of ecosystem processes (14–16). However, the validity of extrapolating these studies to the field is questionable given the low species numbers of soil fauna and plants (if present) typically used, the artificiality of the soil, and the limited number of variables measured. What is required is an approach that manipulates the composition of a soil faunal community with a species richness more akin to that in the field, which includes a multispecies plant community and a reconstructed soil profile and measures the response of a suite of interacting variables. To manipulate the soil community in the field, and maintain it over biologically meaningful temporal and spatial scales, is logistically difficult (17). Ecological microcosms make such investigations eminently more feasible. We used the Ecotron controlled environment facility (18) to test the role of one component of soil community composition—namely, assemblages that dif-

fer in animal body sizes—on carbon flux, and microbial and plant community composition and abundance.

We constructed 15 terrestrial microcosms over a period of 7 months (19) as analogs of a temperate, acid, sheep-grazed grassland (a habitat that occurs widely across the upland regions of northern Britain). We maintained the microcosms in the Ecotron under constant environmental conditions (19) for a further 8.5 months. Soil, plants, fauna, and microorganisms for microcosm construction were collected from the grassland (19). Soil fauna were assigned to a functional group according to body width (20, 21) of the adult or, if the adult was not soil dwelling, largest larval stage. Body size provides a good functional classification because it correlates with metabolic rate, generation time, population density, and food size (22).

¹Natural Environment Research Council Centre for Population Biology, Department of Biological Sciences, Imperial College at Silwood Park, Ascot, SL5 7PY, UK. ²Biodiversity and Ecological Processes Research Group, Cardiff School of Biosciences, Cardiff University, Post Office Box 915, Cardiff, CF10 3TL, UK. ³Department of Biological Sciences, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster, LA1 4YQ, UK. ⁴Centre for Ecology and Hydrology, Merlewood, Grange-over-Sands, LA11 6JU, UK. ⁵Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK. ⁶Technische Universität Darmstadt, Fachbereich 10, Biologie, D-64287 Darmstadt, Germany. ⁷Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK. ⁸School of Biological Sciences, Royal Holloway, University of London, Egham, TW20 0EX, UK. ⁹Macaulay Land Use Research Institute, Craigiebuckler, Aberdeen, AB15 8QH, UK. ¹⁰Institute of Soil Science, University of Hohenheim, 70599 Stuttgart, Germany. ¹¹Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD, UK. ¹²Department of Ecological and Environmental Sciences, University of Helsinki, Niemenkatu 73, FIN-15140 Lahti, Finland. ¹³Department of Biology, University of York, Post Office Box 373, York, YO10 5YW, UK. ¹⁴Natural Environment Research Council, Polaris House, North Star Avenue, Swindon, SN2 1EU, UK.

*To whom correspondence should be addressed. E-mail: m.a.bradford@ic.ac.uk
†Present address: Engineering and Physical Sciences Research Council, Polaris House, North Star Avenue, Swindon, SN2 1ET, UK.
‡Present address: Plant Research Department, Risø National Laboratory, Postbox 49, Roskilde, DK-4000, Denmark.